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14. ABSTRACT

The central objective of this proposal was to test the hypothesis that UV irradiation facilitates the exposure of the HU177 cryptic collagen epitope which may represent an early "solid state biomechanical initiation switch" that promotes inflammation, skin damage and the creation of a melanoma permissive niche. We made significant progress towards the overall goals of our proposal. Our current findings suggest that UVA and UVB dose dependently and differentially trigger conformational changes in collagen type-I and IV resulting in the exposure of the HU177 cryptic epitope. Given the differential exposure of the HU177 cryptic epitope following UV-irradiation, it is possible that distinct differences might be observed in the ability of different cell populations to attach to collagen following UVirradiation. Our findings indicated that while melanoma cell adhesion was generally enhance by 40% to 50% on collagen type-I and IV following UV-irradiation, fibroblast adhesion to collagen type-I was only minimally enhanced. In contrast, dramatic enhancement (5-fold) of macrophage adhesion to UVB irradiated collagen type-IV was observed while little change in adhesion was observed to UVB irradiated collage type-I. These studies suggest that distinct cell types exhibit different adhesive responses to UVA and UVB irradiated collagen type-I and -IV in vitro.

15. SUBJECT TERMS

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Introduction: The central objective of this proposal was to test the hypothesis that UV irradiation facilitates the exposure of the HU177 cryptic collagen epitope which may represent an early "solid state biomechanical initiation switch" that promotes inflammation, skin damage and the creation of a melanoma permissive niche. To examine this hypothesis, we have proposed to assess whether selective targeting of this biomechanical initiation switch prevents or reduces UV-induced inflammation and to examine whether it represents a novel therapeutic approach to prevent and/or reduce melanoma growth. In this regard, three specific aims were proposed. First, we will characterize the kinetics of UV-induced exposure of the HU177 cryptic epitope in vitro and in vivo. These studies will be carried out using a combination of in vitro biochemical assays, ELISAs and immunohistochemical analysis of UV irradiated-collagen as well as the basement membrane preparation Matrigel. In the second aim, we will evaluate the impact of UV-induced biomechanical alterations in collagen and the basement membrane preparation Matrigel has on inflammatory cell, dermal fibroblast, and melanoma cell adhesion, migration, invasion and proliferation as compared to non UV-irradiated ECM. Finally, we will determine the biological consequences of UV-induced exposure of the HU177 cryptic epitope has on inflammatory cell infiltration and on the ability of melanoma cells to establish tumors in vivo.

Body: We have made significant progress towards the overall goals of our proposal during the initial funding period (September 2010 through September 2011). A detailed summary of the research accomplishments as they pertain to the tasks outlined in the statement of work is provided below. Our previous studies have suggested that during invasive cellular processes such as angiogenesis and tumor progression, structural remodeling of extracellular matrix (ECM) components such as collagen and laminin can occur (1-4). These unique physical alterations in the three-dimensional structure of ECM proteins may represent a mechanism to initiate triggering of a biomechanical switch that allows multiple cell types within the tissue microenvironment to physically interact with cryptic epitopes that are inaccessible under normal quiescent physiological conditions (1-4). To this end, previous studies have suggested that ultra violet (UV) waveband exposure can result in biomechanical changes in the physical properties of collagen, which represent nearly 90% of the ECM protein in most tissues including the skin (5-7). Solar irradiation that reaches the earth's surface is largely composed of UVA (320-400nm) and UVB (290-320nm) with UVA comprising the majority (80%-90%) of the UV waveband that reach sun exposed areas of the body. While many studies concerning the effects of UV irradiation have focused on the impact of UVB on DNA, much less is known concerning the effects of UV-irradiation on the non-cellular tissue microenvironment especially as it relates to malignant tumor progression.

Summary research accomplishments

UV-mediated exposure of the HU177 cryptic epitope within collagen in vitro. To begin to assess whether specific UV wavebands my trigger exposure of unique functional epitopes within collagen, we began establishing the experimental conditions to assess the exposure of the HU177 cryptic collagen epitope by ELISA. Our previous studies suggested that UVA irradiation might induce structural changes in basement membrane collagen type-IV. In an initial set of experiments to establish whether UV irradiation could lead to the exposure of the HU177 collagen epitope, microtiter plates coated with either collagen type-I or type-IV and then irradiated with UVA using an Entela UVM-18EL series UV lamp with a maximum 8Watt output. To achieve doses of UVA above 0.6J/cm^2, long exposure times were necessary including some exposure times over 6-12hrs. Extensive experimental analysis indicated that the long exposure times needed to achieve the UV doses resulted in alterations in optical properties of the microtiter wells within which the assays were being conducted. This technical problem prevented us from carrying out further analysis using the UVM-18EL series lamp with immobilized collagen on microtiter plates.

To circumvent this technical issue, we obtained a new UV lamp irradiation system (Tyler Research UV-1) with more accurate and higher power stable UV energy output, which allows shorter exposure periods to achieve the dose range needed. First, we performed a series of experiments to assess the effects of UV irradiation on exposure of the HU177 cryptic epitope within collagen prepared in solution, followed by coating on microtiter plates. Following extensive optimization to insure sensitive detection of the epitope, we

examined the exposure of the HU177 epitope within interstitial collagen type-I over a dose range (0.05J/cm^2-5.0J/cm^2) of UVB (310nm). As shown in figure 1, irradiation of collagen type-I in solution with doses of UVB at 5.0J/cm^2 resulted in exposure of the HU177 cryptic collagen epitope. While a small degree of exposure of the HU177 epitope was detected at different doses, these results were variable. In contrast, little consistent exposure of the HU177 epitope was observed following UVB irradiation of collagen type-I at doses between 0.025J/cm^2 and 5.0J/cm^2. In a similar set of experiments, we assessed the impact of UVA on exposure of the HU177 epitope within interstitial collagen type-I. Interestingly, little consistent exposure of the HU177 epitope was observed within collagen type-I between the UVA doses of 0.1J/cm^2 and 1.0J/cm^2. While exposure of the HU177 epitope was detected within collagen type-I at doses of 2.0cm^2 and 5.0J/cm^2, this exposure was inconsistent and variable. The variation in the extent of exposure of the HU177 cryptic epitope in collagen type-I may be a result of time and/or temperature dependent refolding of the triple helical confirmation of the collagen in solution under the experimental conditions tested.

Figure 1.

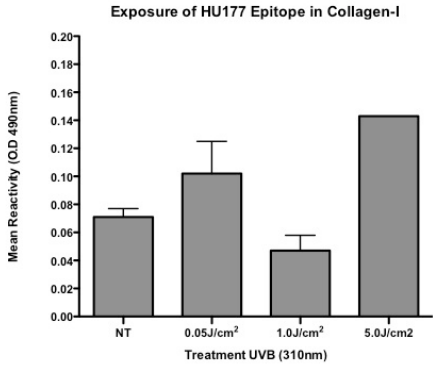


Figure 1. UV-mediated exposure of the HU177 cryptic collagen epitope within collagen in vitro. Human collagen type-I (1.0mg/ml) was either untreated or irradiated with UVB over the dose range indicated and collagen was coated on microtiter wells (0.5ug/ml). Exposure of the HU177 cryptic epitope was detected by solid phase ELISA. Data bars represent the mean Mab D93 reactivity (Optical Density O.D) ± standard deviations from triplicate wells.

UV-mediated exposure of the HU177 cryptic epitope within collagen type-IV in vitro. Using a similar set of experimental approaches as describe above, we examined the exposure of the HU177 epitope within basement membrane collagen type-IV over a dose range (0.05J/cm^2-5.0J/cm^2) of UVA (365) and UVB (310nm) irradiation. As shown in figure 2A, irradiation of collagen type-IV with doses of UVA at 1.0Jcm^2 and 5.0J/cm^2 resulted in extensive exposure of the HU177 cryptic collagen epitope, while little consistent exposure was detected at 0.5J/cm^2. Interestingly, exposure of the HU177 epitope was also detected within collagen type-IV following UVB irradiation at a dose of 1.0J/cm^2 but surprisingly, not at lower doses (0.05J/cm^2) or higher doses (5.0J/cm^2) (Figure 2B). The narrow dose range of UVB capable of exposing the HU177 epitope within collagen type-IV suggests that specific doses of UV-irradiation may cause limited conformational changes or may destroy the epitope under the experimental conditions tested. Again variation in the extent of exposure was observed especially at low (<0.1J/cm^2) and high doses (> 5.0J/cm^2). As mentioned above, the variation in the extent of exposure of the HU177 cryptic epitope may be a result of time and/or temperature dependent refolding of the triple helical confirmation of the collagen in solution under the experimental conditions tested.

Figure 2A. **B.**

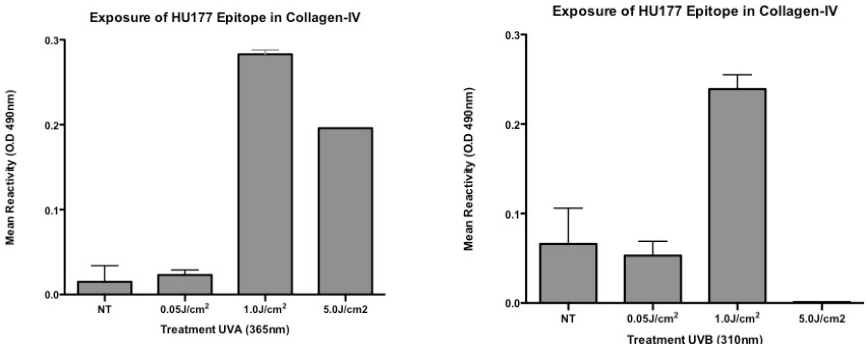
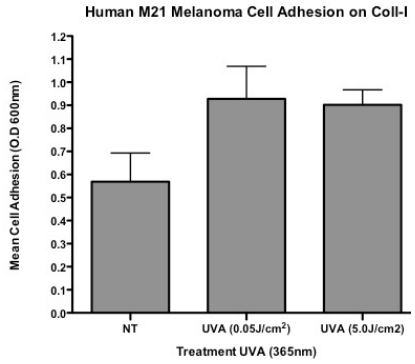


Figure 2. UV-mediated exposure of the HU177 cryptic collagen epitope within collagen in vitro. Human collagen type-IV (1.0mg/ml) was either untreated or irradiated with UVA (A) or UVB (B) over the dose range indicated and collagen was coated on microtiter wells (0.5ug/ml). Exposure of the HU177 cryptic epitope was detected by solid phase ELISA. Data bars represent the mean Mab D93 reactivity (Optical Density O.D) ± standard deviations from triplicate wells.

Alterations of melanoma cell interactions with UV-irradiated collagen in vitro. Our studies on the effects of distinct UV wavebands on the exposure of the HU177 cryptic collagen epitope indicated that UVA and UVB have differential ability to trigger exposure of the HU177 epitope within both collagen type-I and collagen type-IV. These data suggest that solar irradiation may exhibit a differential capacity to exposure the HU177 epitope within sun-exposed sub-compartments within the skin. However, little is known concerning the impact of UV-irradiated collagen has on the adhesion of distinct cell types. In this regard, we began to examine the effect of UV-mediated exposure of the HU177 epitope might have the capacity of distinct cell types to bind interstitial collagen type-I and basement membrane collagen type-IV. Following extensive testing to establish optimal experimental adhesive conditions, we begin to examine the impact of UV-triggered alterations in collagen structure might have on cellular adhesion to collagen and we initially focused on collagen irradiated with either 0.05J/cm^2 or 5.0J.cm^2. As shown in figure 3A, M21 melanoma cell adhesion to collagen-I was enhanced by nearly 40% following UVA irradiation with a dose as low as 0.05J/cm^2. In similar studies a dose dependent enhancement (greater than 50%) of M21 melanoma cell adhesion to collagen type-I was also observed following UVB irradiation (Figure 3B). To examine whether similar enhancement of melanoma cell adhesion occurs following UV-irradiation of collagen type-IV similar experiments were carried out. As shown in figure 3C, UVA irradiation resulted in a dose dependent enhancement of melanoma cell adhesion to collagen type-IV. Interestingly, while a dose 0.05J/cm^2 of UVA enhanced M21 melanoma cell adhesion to collagen type-IV, low dose (0.05J/cm^2) UVB failed to enhance M21 melanoma cell adhesion to collagen type-IV but 5.0J/cm^2 of UVB enhanced adhesion by approximately 50% (figure 3D). These findings suggest that distinct wavebands of UV irradiation may differentially impact the ability of melanoma cells to interact with distinct types of collagen. Current studies are now underway to examine whether the enhanced melanoma cell adhesion is dependent on specific exposure of the HU177 cryptic collagen epitope.

Figure 3 A



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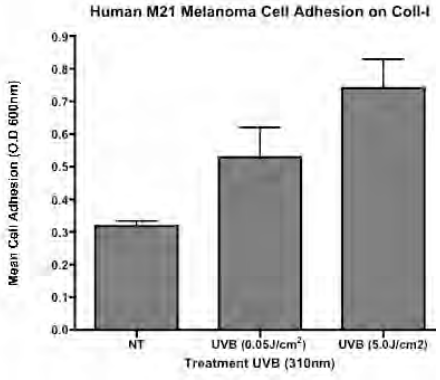

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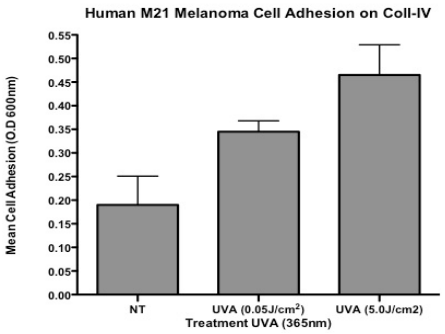

D.

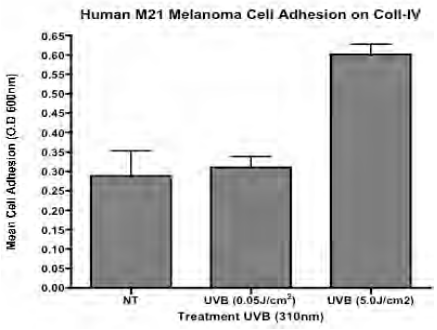

Figure 3. Alterations of melanoma cell interactions with UV-irradiated collagen in vitro. Non-treated and irradiated human collagen type-I (A and B) or collagen type-IV (C and D) were coated on wells and blocked with BSA in PBS. M21 melanoma cells were seeded on the wells and allowed to attach for 15 minutes as we previously described (1-3). Data bars represent mean cell adhesion (Optical Density O.D) $\pm$ standard deviations from triplicate wells.

Alterations of dermal fibroblast cell interactions with UV-irradiated collagen in vitro. The initiation and progression of melanoma has been suggested to involve additional cell types other than the melanoma cells themselves (8-10). Given our studies indicating that melanoma cell interactions with collagen type-I and IV can be differentially and dose dependently enhanced following UV-irradiation, we next examined the effects of UV-irradiated collagen might have on dermal fibroblast cell adhesion. Using similar experimental approaches we coated microtiter wells with UVA or UVB irradiated collagen type-I or type-IV and examined human dermal fibroblast cell adhesion. As shown in figure 4A, fibroblast adhesion to collagen type-I following either low dose (0.05J/cm^2) or a high dose (5.0J/cm^2) UVA irradiation was only slightly (approximately 22%) enhanced as compared to adhesion to non-irradiated collagen. A similar approximately 25% enhancement of fibroblast adhesion was observed on collagen type-I following UVB irradiation at either low or high dose UVB (figure 4B). In contrast to the small increase in fibroblast adhesion to UV-irradiated collagen type-I, UVA irradiation of collagen type-IV at a dose of 5.0J/cm^2 enhanced fibroblast adhesion to collagen IV by over 40% as compared to non-irradiated collagen (Figure 4C). A similar increase (approximately 40%) in fibroblast adhesion to type-IV collagen was also observed following UVB irradiation at a dose of 5.0J/cm^2 (figure 4D). These data are consistent with our previous results indicating the UV-irradiation results in differential and dose dependent alterations in cell adhesion to distinct forms of collagen. Current studies are continuing to examine whether the enhanced fibroblast cell adhesion to collagen type-IV is dependent on specific exposure of the HU177 cryptic collagen epitope.

Figure 4A. **B.**

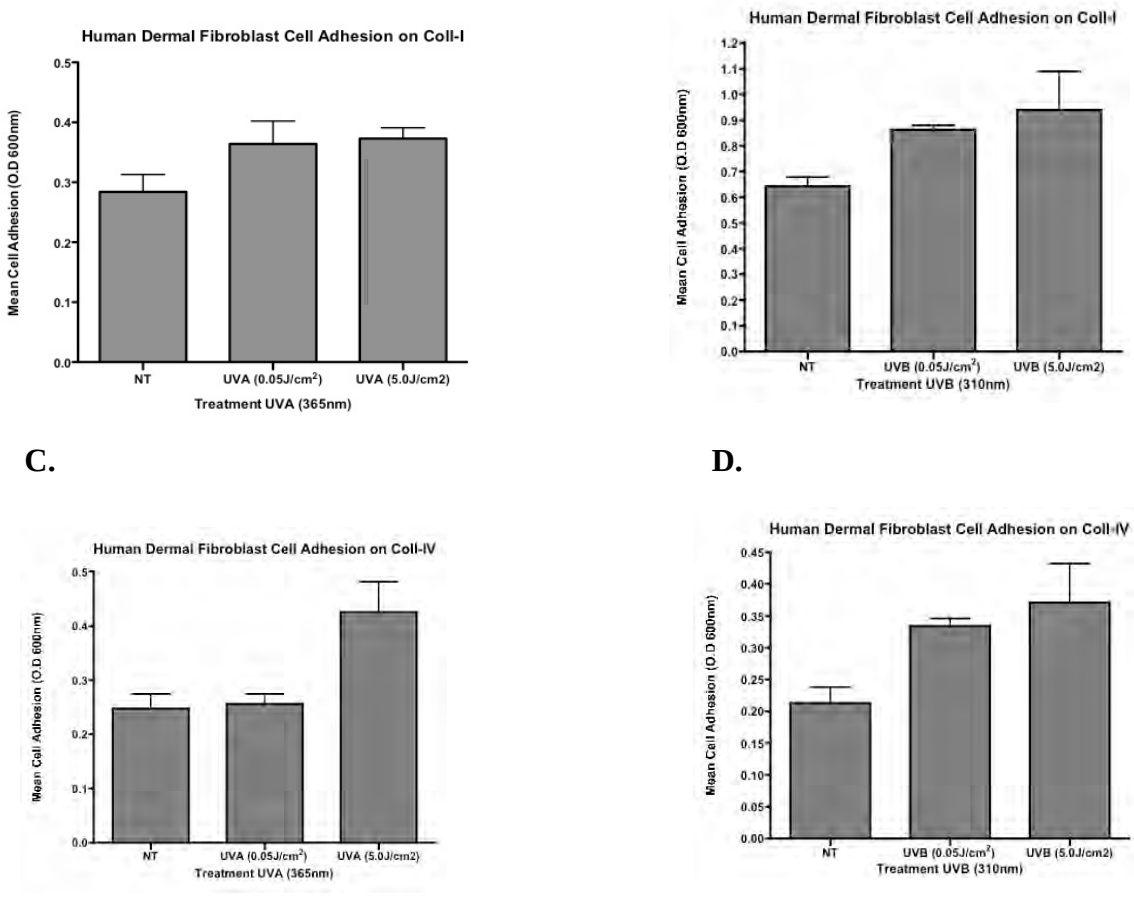

Figure 4. Alterations of fibroblast interactions with UV-irradiated collagen in vitro. Non-treated and irradiated human collagen type-I (A and B) or collagen type-IV (C and D) were coated on wells and blocked with BSA in PBS. Human dermal fibroblasts were seeded on the wells and allowed to attach for 15 minutes as we previously described (1-3). Data bars represent mean cell adhesion (Optical Density O.D) $\pm$ standard deviations from triplicate wells.

Alterations of macrophage interactions with UV-irradiated collagen in vitro. To further examine the impact of UV-irradiation of collagen has of adhesion of distinct cell types that may play a role in melanoma tumor initiation and progression, we evaluated the effects of UV-irradiation has on adhesion of macrophage cell line (Raw 264.7) to collagen type-I and collagen type-IV. In a preliminary experiment collagen type-I and type-IV was irradiated with UVB at a dose of 5.0J/cm^2 and adhesion of murine RAW 264.7 macrophages was assessed. As shown in figure 5A, UVB-irradiation (5.0J/cm^2) had minimal if any effect on macrophage cell adhesion to collagen type-I as compared to non-irradiated collagen. Interestingly, similar UVB irradiation (5.0J/cm^2) of collagen type-IV dramatically enhanced macrophage adhesion by over 5-fold as compared to non-irradiation collagen (figure 5B). While clearly preliminary, these data suggest that UVB irradiation of collagen type-IV, but not collagen type-I may result in a dramatic enhancement of macrophage adhesion to basement membrane collagen type-IV. Additional experiments will be needed to confirm these studies.

Figure 5A. **B.**

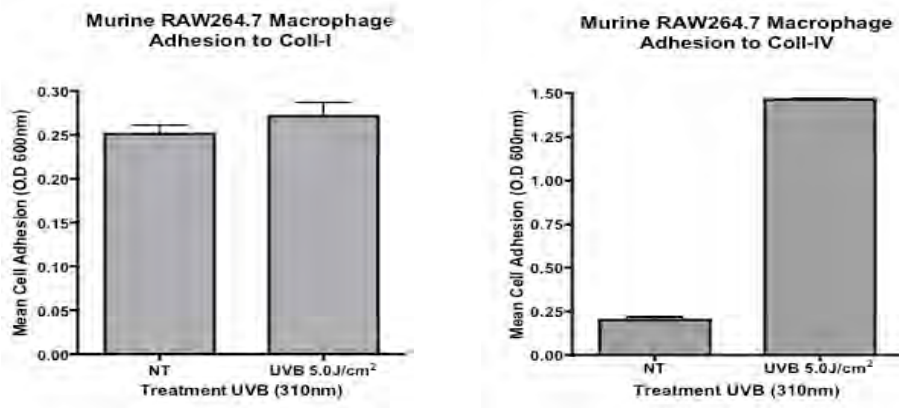

Figure 5. Alterations of macrophage interactions with UV-irradiated collagen in vitro. Non-treated and irradiated human collagen type-I (A) or collagen type-IV (B) were coated on wells and blocked with BSA in PBS. Murine RAW 264.7 macrophage were seeded on the coated wells and allowed to attach for 15 minutes as we previously described (1-3). Data bars represent mean cell adhesion (Optical Density O.D) $\pm$ standard deviations from triplicate wells.

UV-mediated exposure of the HU177 cryptic collagen epitope within the skin in vivo. In our previous preliminary studies, UVA irradiation of murine skin resulted in exposure of the HU177 cryptic collagen epitope. Given the technical problems observed with the original Entela UVM-18EL series UV lamp, we obtained the new Tyler Research UV-1 irradiation system with more accurate and higher power stable UV energy output. Studies are currently underway to establish conditions for using the new UV irradiator to deliver UVA and UVB irradiation over a broad dose range to the skin of mice. In an initial preliminary study of UVA and UVB at a dose of 0.5J.cm^2 the HU177 cryptic epitope appeared exposed 24hrs following UV irradiation. Studies are currently underway to optimize dose delivery and immunofluorescence detection of the epitope within these UV-irradiated skin tissues.

Key Research Accomplishments:

1). UVA and UVB irradiation dose dependently trigger conformational changes in both collagen type-I and collagen type-IV resulting in the exposure of the HU177 cryptic epitope.

2). The relative exposure of the HU177 epitope within each specific type of collagen varied and depended on the type of collagen and the dose of distinct wavebands of UV-irradiation.

3). Human M21 melanoma cell adhesion to collagen type-I and IV was enhanced (40%-50%) following UVA and UVB irradiation.

4). Human dermal fibroblast cell adhesion to collagen type-I was only minimally (20%-25%) enhanced following UVA or UVB irradiation, while high dose UVA and UVB enhanced fibroblast adhesion to collagen type IV by approximately 40%.

5). Preliminary evidence suggest a dramatic 5-fold increase in macrophage adhesion to collagen type-IV following UVB irradiation, while little if any change in adhesion was observed following UVB irradiation of collagen type-I.

6). Technical and experimental conditions are being established for the new UV-irradiator to allow precise dose dependent studies of the impact of distinct UV-wave bands on exposure of the HU177 epitope in skin in vivo.

Reportable Outcomes: None

Conclusions:
Our experimental findings suggest that UVA and UVB irradiation can dose dependently trigger conformational changes in both collagen type-I and collagen type-IV in vitro resulting in the exposure of the HU177 cryptic collagen epitope. Importantly, the relative exposure of the HU177 epitope within each type of collagen varied and depended on the type of collagen and the dose of distinct wavebands of UV-irradiation used. These studies taken together suggest the possibility that the structural conformations of distinct types of collagen within the skin tissue microenvironment may be differentially altered following solar irradiation and the relative levels of exposure of the HU177 cryptic collagen epitope my vary depending on the ratio of the UVA/UVB wavebands and the type of collagen exposed. Given the clear differential exposure of the HU177 epitope observed in vitro, it is likely that distinct differences will be observed in adhesive behavior of cells to collagen irradiated by distinct wavebands of UV irradiation. In this regard, our experimental findings indicated that distinct cell types known to be present within the skin tissue microenvironment exhibit differential ability to interact with UV-irradiated collagen type-I and type-IV. In particular, while melanoma cell adhesion was generally enhance by 40% to 50% on collagen type-I and IV following UVA and UVB irradiation, fibroblast cell adhesion to collagen type-I was only minimally enhanced. Importantly, a dramatic enhancement (over 5-fold) of macrophage adhesion to UVB irradiated type-IV was observed while little change in macrophage adhesion was observed to UVB irradiated collage type-I. Taken together, these novel studies suggest that distinct cell types exhibit different adhesive properties to UVA and UVB irradiated collagen type-I and collagen type-IV.

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Appendices: none